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GRANT NO: DAMD17-94-J-4398

TITLE: Targeted Gene Delivery to Accomplish Gene Therapy for Breast Cancer

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REPORT DATE: August 28, 1995

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	28 August 95	Annual 1 Aug 94 - 31 Jul 95

4. TITLE AND SUBTITLE
Targeted Gene Delivery to Accomplish Gene Therapy for Breast Cancer

5. FUNDING NUMBERS
DAMD17-94-J-4398

6. AUTHOR(S)
David T. Curiel, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Alabama at Birmingham
Birmingham, Alabama 35294-3300

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

We are developing methods to derive a gene transfer vector capable of accomplishing targeted gene delivery to metastatic breast cancer cells. In this regard, strategies were employed to modify adenoviral vectors by altering their binding tropism. Genetic methods undertaken allowed for the modification of the native adenoviral binding protein (fiber) to incorporate cancer-relevant cell-binding ligands. Immunologic methods yielded an antifiber antibody which specifically ablates native adenoviral tropism and provides a site for the subsequent addition of breast cancer-relevant ligands. Chemical methods demonstrated that biotin could be added to the virion exterior as an anchor for a streptavidin bridge to allow for incorporating of breast cancer relevant ligands. The results developed herein have allowed for the successful modification of the adenoviral vector to allow it to accomplish cell-specific gene delivery. This important first step will now allow the evaluation of this vector system in the context of in vitro and in vivo models of breast cancer. The utility of the vector in this context will allow the development of gene therapy strategies for disseminated breast cancer on this basis.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Gene therapy, vector, receptor, adenovirus Breast Cancer	12 16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

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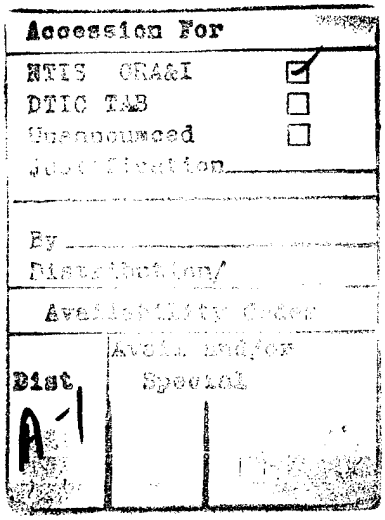

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PI - Signature Date

Annual Report for Grant Number DAMD 17-94-J-4398

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(5). Introduction

It is the purpose of the present proposal to derive a gene transfer vector capable of specific and selective transduction of disseminated neoplastic breast cancer cells as a first step towards the development of gene therapy strategies for carcinoma of the breast. The development of a vector with this capacity would allow the targeting of malignant cells *in situ* after systemic delivery. Capitalizing on the unique capacity of recombinant adenoviral vectors to accomplish high efficiency *in vivo* gene delivery, it is our hypothesis that further modifications of this vector may be achieved to alter parent virus tropism such that selective transduction of malignant cells may be accomplished. These modifications would allow entry of the vector into receptor-mediated endocytosis pathways characteristic of the target breast cancer cells. To investigate mechanisms by which adenoviral tropism could be modified, several molecular strategies were employed. In this regard, the initial strategy to derive a tropism-modified recombinant adenovirus was directed towards genetic modification of the fiber protein to accomplish incorporation of heterologous cell-binding ligands, which could then mediate adenoviral entry via alternate receptor pathways. This approach capitalized on the knowledge that the endogenous cell-binding ligand of adenovirus was localized within the knob portion of the fiber protein. The aim was thus to localize the novel cell-binding ligand in the analogous position. This would accomplish two goals: 1) the novel cell-binding ligand would be localized in the region of the endogenous ligand, likely a propitious site as relates to interaction with the cognate cellular receptor; and 2) the novel cell binding ligand would be removed from other adenoviral capsid proteins, whose function might be important in distal, post-binding entry functions. The incorporation of heterologous peptides in the context of the fiber protein required consideration of the strict structural limitations of the fiber quaternary configuration. In this regard, the fiber protein is synthesized initially as a monomer [1]. Upon localization to the nucleus, the molecule trimerizes by virtue of intramolecular, non-covalent interactions, initiated at the carboxy-terminus of the molecule. After trimerization, the amino terminus of the native fiber can then insert into the penton base [2]. Thus, additions to the knob portion of the fiber, corresponding to the carboxy terminus of the molecule, could potentially impair trimer formation and thus prevent incorporation of chimeric fiber molecules into the mature adenoviral capsid. In addition to these considerations, it was important to achieve a final quaternary configuration whereby the incorporated ligand was localized on the exterior of the mature fiber trimer. Hence, it was not apparent *a priori* that added ligands would be localized outside the molecular structure of the knob and thus accessible to achieve target cell binding. With these considerations in mind, a strategy was undertaken to create fiber-ligand fusion proteins by genetically incorporating into the fiber gene heterologous sequences encoding peptides with physiologic ligand functions (Figure 1).

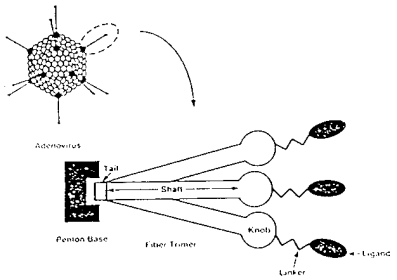

Figure 1. Strategy to incorporate heterologous peptide ligand at the knob domain of adenoviral fiber. The strategy presented herein involves the genetic modification of the fiber gene to generate a fiber chimera consisting of mature trimeric fiber, peptide linker, and an added physiologic ligand at the knob domain. This ligand is designed to target the adenovirus into heterologous cellular entry pathways.

The initial analysis needed to confirm that: 1) the fiber fusion genes produced a chimeric fiber molecule capable of maturing into a normal trimeric quaternary configuration; and 2) the fiber fusion genes express a chimeric fiber molecule whereby the heterologous ligand is localized on the exterior of the trimeric molecule. Achievement of these goals, even in a limited context, would predict that further analysis would identify the optimal ligands from the standpoints of cell binding and internalization. The preliminary data related to these goals are described herein:

(6) Body

A. <u>Construction of a fiber gene encoding a peptide ligand.</u> To create adenoviral fiber-peptide ligand fusion proteins, several cloning maneuvers were performed on the 3' end of the adenovirus type 5 fiber gene (figure 2). First, site-directed mutagenesis was performed to introduce a unique BamHI restriction site (denoted as **B** in figure 2) in this region of the gene to facilitate cloning of the test ligand. Second, a flexible ten amino acid (ProSerAlaSerAlaSerAlaSerAlaPro) hinge region (**linker**) was introduced between the

carboxy terminus of the fiber protein and the ligand peptide to minimize any possible steric constraints and to present the test ligand extended away from the body of the fiber protein. Finally, we chose the terminal decapeptide of the gastrin releasing peptide (**GRP ligand**) as the initial test ligand due to its small size and its ability to be internalized into its target cell by a receptor-mediated endocytosis pathway [3,4]. GRP receptors are overexpressed in various carcinomas, making GRP an attractive ligand to test the feasibility of constructing a targeted adenovirus for cancer gene therapy applications.

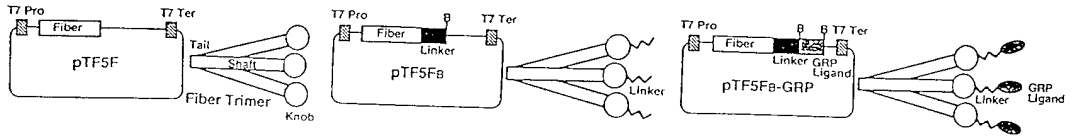

Figure 2. Schematic of fiber-GRP ligand fusion protein construction. Plasmids were constructed to contain wild-type fiber of adenovirus type 5 only (pTF5F), fiber and ten amino acid linker only (pTF5FB), and the fiber-GRP fusion construct (pTF5FB-GRP).

B. <u>Expression of genetically-modified fiber protein.</u> In order to express the above fiber fusion proteins, a vaccinia virus expression system was employed. Specifically, HeLa cells grown in low serum media were infected with a recombinant vaccinia virus containing T7 RNA polymerase prior to Lipofectin mediated transfection of pTF5FB and pTF5FB-GRP, which contain fusion genes driven by the T7 promoter. To confirm the presence of a fiber-GRP fusion product, cell lysates prepared from vaccinia infected HeLa cells which had been transfected with either pTF5FB or pTF5FB-GRP were immunoprecipitated with either a monoclonal anti-fiber antibody (4D2, 2A6, or AF7A) or a rabbit anti-human GRP antibody (α-GRP, DAKOPATTS). 4D2 recognizes fiber monomers and trimers, while 2A6 and AF7A both recognize fiber trimers only.

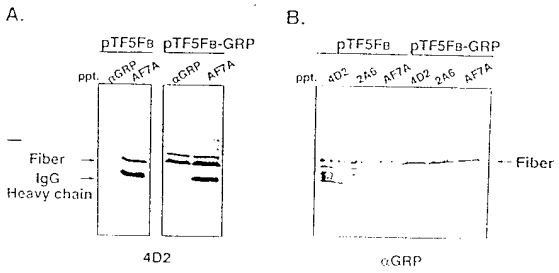

Figure 3. Evaluation of adenoviral fiber protein variants. **A.** HeLa cell lysates transfected with fiber fusion constructs were immunoprecipitated with α-GRP or AF7A antibodies. Western blots were then performed on boiled samples using 4D2 antibody. **B.** HeLa cell lysates transfected with fiber fusion constructs were immunoprecipitated with three anti-fiber antibodies: 4D2, AF7A, and 2A6. Western blots were then probed with an α-GRP antibody.

As shown in figure 3A, the α-GRP antibody immunoprecipitates a protein which both has a size consistent with that of mature fiber and is also recognized by an anti-fiber antibody, 4D2. This immunoprecipitation product was identical in size to the product of immunoprecipitated fiber-GRP fusion protein with an anti-fiber antibody that recognizes fiber trimers only, AF7A. In contrast, when HeLa cell lysates that were transfected with a construct encoding the fiber with the linker only, and no GRP ligand, an α-GRP antibody was unable to precipitate a protein of similar size. This result indicated that the GRP peptide was indeed synthesized as part of a fusion protein with the adenovirus fiber. To confirm this result, HeLa cell lysates which were transfected with a plasmid containing the fiber-GRP fusion gene were first immunoprecipitated with different anti-fiber antibodies, and then subjected to western blot analysis with an α-GRP antibody. Figure 3B indicates that immunoprecipitated fiber-GRP fusion proteins were recognized by an α-GRP antibody. In contrast, when cell lysates containing the modified fiber with the linker and no ligand were immunoprecipitated with different anti-fiber antibodies, subjected to western blot analysis, and probed with an α-GRP antibody, no antibody specific bands could be detected. Thus, these studies demonstrate that the fusion gene construct we have derived is capable of directing the expression of a fiber-GRP fusion protein in a eukaryotic host system.

C. <u>Determination of the quaternary structure of fiber protein variants in vaccinia vector-infected cells.</u> Correct fiber protein folding is absolutely required for incorporation of the fiber protein into the vertices of nascent adenovirus capsids. Since our aim is to construct a recombinant adenovirus with a

genetically modified fiber, it was first important to determine whether incorporation of exogenous peptides at the carboxy terminus of fiber still allowed proper fiber protein folding into the native quaternary configuration. Preservation of the quaternary structure or trimerization of the fiber-ligand fusion protein would theoretically be indicative of proper adenovirus capsid assembly with the modified fiber proteins. To determine the quaternary structure of the fiber-GRP fusion protein, HeLa cell lysates transfected with pTF5F, pTF5FB, or pTF5FB-GRP were subjected to western blot analysis and probed with an anti-fiber antibody 4D2. On SDS-PAGE, boiled fiber protein migrates as a monomer, whereas in unboiled samples fiber migrates as a trimer.

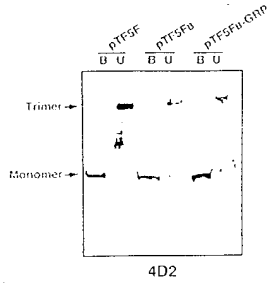

Figure 4. Determination of the quaternary structure of the fiber-GRP fusion protein. Boiled and unboiled HeLa cell lysates transfected with three fiber constructs were analyzed by western blot using 4D2 anti-fiber antibody.

Figure 4 shows that boiled fiber-GRP fusion protein migrated in the monomeric form of the protein whereas unboiled fiber-GRP fusion protein migrated as a trimer. This result indicates that it is possible to add exogenous sequences to the carboxy terminus of the fiber protein, at least as large as twenty-two amino acids, without perturbing the quaternary structure of the protein. This result demonstrates that the derived fiber-ligand fusion gene retains the requisite quaternary configuration characteristics required for its incorporation into assembled adenoviral capsids.

D. <u>Accessibility of the GRP ligand in the native form of the fiber-GRP fusion protein.</u> We have shown that it is possible to add short peptide sequences to the carboxy terminus of the adenovirus fiber protein without impairing either the biosynthesis or the proper folding of the protein. What remained to be determined was whether the carboxy terminus of the fiber protein was internal or external in the native configuration of the fiber protein. With this unanswered, it was difficult to predict whether the GRP ligand would therefore be localized on the fiber protein externally as desired. If the GRP ligand, or any other ligand for that matter, is to be used to redirect adenoviral vectors, it is imperative that the ligand be externally accessible to its cellular receptor. We thus wanted to determine whether the GRP ligand in the trimeric form of the fiber-GRP fusion protein was exposed in its quaternary structure. In an immunofluorescence assay, α-GRP antibody was used to determine the presence of the fiber-GRP fusion protein in the nuclei of transfected cells (data not shown). This confirmed that the biosynthetic route of the fiber-GRP fusion constructs was identical to that of wild-type fiber protein. However, the immunofluorescence assay is unable to distinguish whether the α-GRP antibody bound to the monomeric or trimeric form of the fiber-GRP fusion protein. To answer this question, an immunoblot assay was performed in which boiled and unboiled HeLa cell lysates from cells transfected with pTF5FB or pTF5FB-GRP were probed with either 4D2, which recognizes both fiber trimers and monomers, or α-GRP antibody. In this assay, unboiled lysates should have intact trimeric fiber proteins, while boiling ablates this quaternary structure, resulting in monomers only.

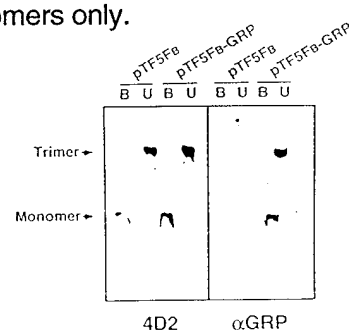

Figure 5. Accessibility of the GRP ligand in the fiber-GRP trimer. To determine whether the GRP ligand in the native form of the fiber-GRP fusion protein was accessible to binding, an immunoblot assay was performed in which boiled (B) and unboiled (U) HeLa cell lysates transfected with either pTF5FB or pTF5FB-GRP were analyzed by SDS-PAGE and probed with anti-fiber antibody 4D2 or α-GRP.

As shown in Figure 5, when boiled and unboiled lysates containing the modified fiber with the linker only (pTF5FB) were subjected to Western blot analysis and probed with 4D2, a monomer and trimer band were detected for the boiled and unboiled samples, respectively. When the same samples were probed with α-GRP antibody, no bands could be detected, due to the absence of ligand in this construct. When boiled and unboiled cell lysates containing the fiber-GRP fusion protein (pTF5FB-GRP) were subjected to the same type of analysis and probed with 4D2, a monomer and trimer band were detected, respectively. When the same samples were probed with an α-GRP antibody, a monomer band could be detected in the boiled sample and a trimer band could be detected in the unboiled sample. These results indicate that not only is the GRP ligand in the fiber-GRP fusion protein accessible to binding in the monomeric form of the protein, but the ligand is also exposed and accessible to binding in the native or trimeric form of the protein. These preliminary studies demonstrated several key properties of the fiber-ligand fusion protein: 1) the protein retains its native configuration; 2) the protein retains its native biosynthetic profile; and 3) the protein presents the added ligand in an exterior, surface exposed localization. These studies thus demonstrate the feasibility of introducing heterologous peptide ligands into the cell-binding domain of the adenoviral fiber protein in a manner consistent with the ultimate derivation of chimeric adenoviral particles. This important first step establishes the potential utility of this line of investigation as a strategy to accomplish tropism-modified adenoviral vectors. The studies proposed herein will allow the further development of this targetable vector system.

From the above discussion, it is apparent that the exact structural limitations for the incorporation of alternate ligands into adenoviral fiber are not known. Thus, while a twenty-two amino acid ligand may be genetically incorporated into fiber without loss of structural integrity and presented on its surface, it is not apparent, *a priori*, that incorporation of larger ligands will also be facilitated in this manner. To circumvent this potential limitation, immunological methods will be used to introduce alternate ligands onto the surface of adenoviral virions. As stated in the background section, it has been shown that antibody-ligand bridging techniques can be used to permit the uptake of retroviruses into non-permissive cells [5,6]. Specifically, retroviruses can be retargeted to infect originally non-permissive cells by immunologically crosslinking them to cell-specific determinants. This is done by incubating target cells in culture with biotinylated anti-surface determinant antibody, crosslinking with streptavidin, adding biotinylated anti-retroviral glycoprotein antibody, and finally adding retrovirus. Thus, an "immunological bridge" was constructed *in vitro* by the sequential addition of each component to target cells. In light of these findings, we hypothesized that utilization of neutralizing monoclonal antibodies to the knob domain of adenoviral fiber would facilitate construction of tropism-modified adenoviral vectors in an analogous manner. In addition, this strategy would satisfy both criteria necessary for construction of tropism-modified adenoviral vectors: ablation of endogenous binding and introduction of alternate ligands. Yet from the above experimental design, it is obvious that construction of such an "immunological bridge" is not amenable to *in vivo* systemic usage. Instead, we hypothesized that generation of anti-fiber antibody/ligand fusion protein would serve an analogous purpose for retargeting adenoviral tropism, but would also facilitate *in vivo* use of such a virus. To facilitate construction of this fusion protein, single chain variants of antibodies directed to the knob domain of adenoviral fiber protein (anti-knob sFv) can be genetically constructed. Genetic constructs can then be made to generate anti-knob sFv/ligand fusion proteins, as well as anti-knob sFv/anti-cell surface receptor sFv fusion proteins. These genetic constructs would then circumvent utilization of chemical crosslinking mechanisms in the generation of tropism modified adenoviral vectors. In this regard, a strategy was initiated to investigate construction of immunological bridges involving the adenoviral fiber protein.

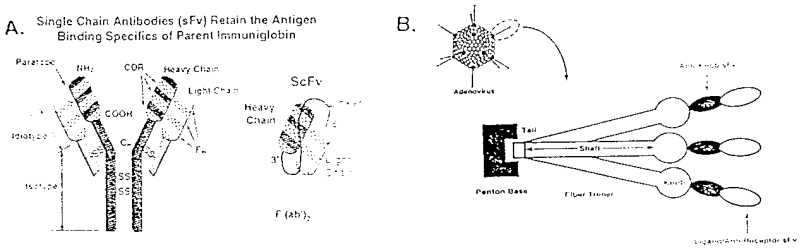

Figure 6. Schematic for construction of adenovirus-antibody-ligand immunological bridges. This strategy involves the use of an anti-knob sFv/ligand or anti-knob sFv/anti-receptor sFv fusion protein to immunologically redirect adenoviral tropism into heterologous cellular pathways.

E. <u>Production and characterization of anti-knob monoclonal antibodies.</u> As the first step towards creating immunological bridges involving the adenoviral fiber protein, murine monoclonal antibodies against recombinant knob were generated. It has been shown that the cell binding domain of adenovirus fiber

protein is localized within its knob domain. Thus, monoclonal antibodies to this domain would theoretically allow the following: ablation of endogenous binding and introduction of a specific, localized cell binding domain which could facilitate modification of adenoviral tropism. This location of the "immunological bridge" would likely be a propitious site with regards to receptor binding. In addition, utilization of antibodies to the knob domain would potentially avoid interference with the function of other adenoviral capsid proteins, which are necessary for post-receptor binding events. Towards this end, mice were first immunized with intact adenovirus, followed by two rounds of immunization with purified recombinant knob protein (provided by R. Gerard, see letter of collaboration). Monoclonal hybridomas were generated by standard techniques. Supernatants from these hybridomas were assayed for the following phenotypic characteristics: isotype, reactivity with recombinant knob, and neutralization of adenoviral cytopathic effect (CPE). Isotype was determined by the commercially available IsoStrip assay (Boehringer Mannheim). Reactivity with recombinant knob was assayed by ELISA using plates coated with recombinant adenovirus type 5 knob. Extent of antibody binding was determined photometrically with an anti-IgG-alkaline phosphatase conjugated antibody. Neutralization of adenovirus type 5 cytopathic effect was determined by inhibition of adenovirus infection of HeLa cells. The neutralization capacity, or relative strength of inhibition of adenovirus infection, was assigned for each clone on a scale from + to +++.

Anti-Fiber Knob Antibody Supernatants

Clone	Neutralization*	Isotype	Knob Reactivity**
1D6.1	++	IgG2a κ	1.124
1D6.2	++	IgG2a κ	1.002
1D6.3	+++	IgG2a κ	0.897
1D6.8	++	IgG2a κ	0.778
1D6.13	++	IgG2a κ	0.912
1D6.14	+++	IgG2a κ	0.941
2A4.1	+	IgG2a κ	1.244
7A2.1	+	.....	0.925
7A2.6	+	.....	0.908
7A2.7	+++	IgG2a κ	0.766
7A2.10	+	.....	0.786
7A2.13	+	.....	0.728
8C2.2	++	IgG2a κ	1.153
8C2.13	+	.....	0.921
8C2.15	+	.....	1.161
8D4.6	+	IgG2a κ	0.745

*Neutralization of Type 5 Adenovirus
+++ = most
++ = good
+ = good

**Reactivity to Type 5 Fiber Knob Using an ELISA Assay

Figure 7. Phenotypic characteristics of antibody supernatants from anti-knob monoclonal hybridomas.

As shown in figure 7, several clones were obtained which bind recombinant knob with high affinity and exhibit neutralization of infectivity on HeLa cells of adenovirus type 5. From this group, clones 1D6.3, 1D6.14, and 7A2.7 were chosen for further study, since they exhibit the phenotypic characteristics important in modifying adenoviral tropism via immunological crosslinking. From the ELISA data, it is evident that these three antibodies bind knob protein with high affinity. In addition, as determined by the inhibition of infectivity of adenovirus, it is evident that these antibodies are localized within a region of the knob domain essential for adenovirus entry. Based upon these promising results, these clones will be further characterized by Pierre Boulanger (see letter of collaboration) to determine the exact location of binding within the knob region of adenovirus fiber. In parallel with characterization of these neutralizing anti-knob monoclonal antibodies, we will derive the corresponding single chain immunoglobulin variant (sFv). This maneuver will yield cDNAs of sFv's derived from the respective parent antibody. This will allow the genetic incorporation of receptor ligands or anti-receptor sFvs into immunological fusion proteins, which will then be used to immunologically retarget adenoviral tropism. In regards to the construction of sFv molecules, we have recently described the development of an anti-erbB-2 sFv in the context of the mutation compensation technique of intracellular immunization for ovarian cancer [7]. In addition, we have developed sFv's against p53 and MHC class II antigens employing the phage display library technique directly from the corresponding hybridomas. Thus, we possess the capacity to readily derive sFv's in our laboratory. On the whole, these studies represent the first step in the construction of immunological bridges designed for the modification of adenoviral tropism.

As an alternative to genetic and immunological methods to modify adenoviral tropism, chemical methods were also investigated. The feasibility of this approach was demonstrated by Neda et al., who showed that chemical modification of retroviral virions by addition of carbohydrates facilitated ecotropic Moloney murine leukemia virus (MoMLV) infection of human hepatocytes via the asialoglycoprotein receptor [8]. In this context, we hypothesized that adenoviral virions could be directly chemically modified with biotin, which would facilitate incorporation of biotinylated ligands or antibodies via a streptavidin bridge. These biotinylated ligands or anti-receptor antibodies could then redirect the adenovirus through heterologous cell entry pathways. This method would then obviate antibody-ligand binding affinity considerations involved in construction of immunological bridges, since a covalent modification would facilitate incorporation of the first component of the multifunctional complex. A biotinylation reagent which covalently links to free amine groups on target proteins was chosen. This reagent facilitates covalent attachment via primary amines of target proteins within the adenovirus capsid. In addition, a reagent was chosen which contains an eleven carbon spacer arm, which would reduce steric hindrance between biotinylated molecules within a single

avidin bridge. Thus, although this method would allow introduction of a potentially more stable molecular conjugate on the adenoviral capsid, functional consequences of the chemical modification were an important parameter. This method mediates direct modification of adenoviral capsid proteins in a non-specific manner and could potentially impair their biological function. With these considerations in mind, the following experiments were done.

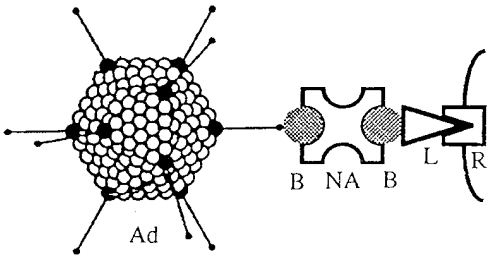

Figure 8. Schematic of biotinylated adenoviral capsid conjugates. Biotinylation of adenoviral capsids results in covalent modification of all capsid protein and facilitates the incorporation of biotinylated ligands into the conjugate via avidin bridge. This maneuver should then confer modified adenoviral tropism.

F. <u>Biotinylation of adenoviral capsids.</u> The first step taken in the chemical modification of adenoviral capsids was direct biotinylation of capsid proteins. In this regard, a variety of biotinylation reagents exist which can target specific functional groups or specific amino acids within proteins. Thus, a particular reagent may be chosen which may preserve function of the target protein. The initial reagent chosen for biotinylation of adenoviral capsids was NHS-LC-biotin (Pierce), an N-hydrosuccinimide biotin ester. This water-soluble reagent covalently reacts with free primary amines present on target proteins and contains an eleven carbon linker arm (22.4 Å) to reduce steric constraints between biotinylated proteins within a single streptavidin bridge [9]. To investigate the efficacy of this primary amine targeted biotinylation reagent, it was first necessary to determine if this reagent could facilitate biotinylation of potential free amines on adenoviral capsid proteins. Adenoviral virions were thus modified under varying biotinylation conditions to determine if reaction conditions correlated with the degree of biotinylation of the capsid. To this end, adenoviral particles were incubated with 10 fold dilutions of biotin-NHS (10 mM to 10 μM)). Two-fold dilutions of these virions were then blotted onto nitrocellulose filters and assayed by dot blot using a streptavidin-alkaline phosphatase (AP) conjugate to detect biotinylated adenoviral proteins.

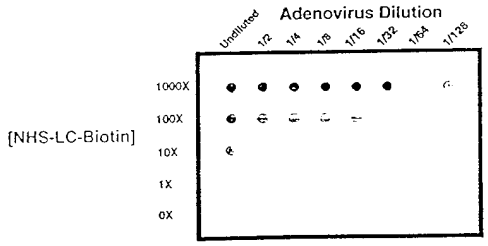

Figure 9. Dot blot of biotinylated adenoviral capsids. Two-fold dilutions of each biotinylation reaction (0X to 1000X) were blotted on nitrocellulose filters and probed with streptavidin-AP conjugate.

As shown in figure 9, it is possible to chemically modify adenoviral capsid proteins with biotin. Thus, free amine groups present on N-terminal amino acids or the ε-amino group of lysine are present on the surface of adenovirus to facilitate chemical conjugation with NHS-LC-biotin. Biotinylation was achieved over the range of 1 μg/ml to 1 mg/ml, as seen by the positive blot in lane A. Furthermore, the degree of biotinylation correlates with the biotinylation conditions. Increasing amounts of biotin could be easily detected on the surface of the adenovirus at increasing dilutions. Specifically, biotinylation could be detected at the 1:128 dilution of adenovirus for both the 100X condition, as well as the 1000X condition. Thus, a plateau effect is achieved at high concentration of biotin, indicating a saturation of free amine groups on target proteins. In light of the fact that adenoviral proteins can be effectively biotinylated, the effects of this chemical modification on the biological properties of adenovirus must be determined.

G. <u>Biotinylation occurs on all adenoviral capsid proteins.</u> As mentioned above, NHS-LC-biotin mediates biotinylation of primary amines on target proteins. Due to the ubiquitous nature of this chemical reaction, it was necessary to determine the localization of the biotinylation on capsid proteins. Thus, the 1:2 dilution of the 100X biotinylated adenovirus was analyzed on denaturing SDS-polyacrylamide gel electrophoresis using standard methods. Biotinylation of capsid proteins was then assayed by probing with avidin conjugated alkaline phosphatase (data not shown). This experiment showed a ladder of

bands, indicating all adenoviral capsid proteins were biotinylated. This was the expected result due to the ubiquitous presence of lysine residues within the capsid proteins.

H. <u>Biotinylation of adenoviral capsids does not impair gene transfer.</u> Due to the ubiquitous action of biotin-NHS with primary amines and its efficient reaction with the adenoviral capsid, it was not apparent, *a priori*, whether this maneuver would destroy biological function of capsid proteins and thus render modified virions non-infectious. Theoretically, biotinylation could occur on amino acids critical to the function of capsid proteins such as fiber, which is necessary for receptor binding, and penton base, which is necessary for post-receptor internalization. To determine if biological activity of modified capsids was impaired, the effects of biotinylation on the ability of recombinant adenovirus to mediate gene transfer was determined. Specifically, recombinant adenovirus containing the luciferase reporter gene driven by the CMV promoter (AdCMVLuc) was biotinylated with 10 fold dilutions of biotin-NHS as above. This chemically modified virus was then used to transfect SKOV-3 human ovarian carcinoma cells, a cell line known to be highly transducible by adenoviral vectors. Transfections were performed in quadruplicate with the various adenoviral lots. Twenty-four hours post infection, gene transfer was assayed for luciferase expression by luminometer.

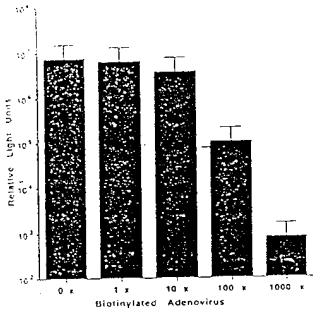

Figure 10. Luciferase expression in SKOV-3 cells transfected with biotinylated recombinant AdCMVLuc adenovirus which contains the luciferase reporter gene.

As shown in figure 10, biotinylation of adenoviral capsids does not prevent gene transfer properties of the recombinant adenovirus vector. As shown in the previous section, the degree of biotinylation of adenoviral capsids directly correlates with biotinylation conditions. With increasing amounts of biotin-NHS, adenoviral capsid proteins are biotinylated to a greater extent. From the above experiment, it can be concluded that with increasing extent of biotinylation, gene transfer efficiency, as monitored by luciferase expression, decreases. A critical threshold is reached whereby biological activity of the adenovirus is compromised. This level is obtained at the 1000X (10 mM) condition, which correlates with the saturation point of biotinylation as demonstrated in figure 10. Importantly, even with relatively high biotinylation conditions (100 μM), high levels of luciferase expression are still obtained (10^5 RLU). Levels of expression for both 10 μM and 1 mM were of the same magnitude (10^7). Thus, even relatively high biotinylation conditions allows infectious entry of adenoviral virions, confirming maintenance of biological integrity of capsid proteins. It is only at the condition corresponding to 10 mM of biotin-NHS, that chemical modification critically impairs either pre- or post-receptor binding events and prevents AdCMVLuc mediated reporter gene transfer. Thus, a critical threshold is reached whereby direct biotinylation prevents biological function of adenovirus. On the whole, these studies represent the important first step in the construction of biotin/avidin crosslinked tropism-modified adenovirus vectors.

(7). Conclusions

Three distinct strategies to develop a targeted gene therapy vector based upon recombinant adenoviral vectors have been initiated. The accomplishments in each effort have been summarized in the corresponding sections preceding. This significant progress will shortly allow analysis of targeted gene delivery in the context of *in vitro* and *in vivo* models relevant to disseminated carcinoma of the breast.

(8). References

1. Sundquist, B., E. Everitt, L. Philipson, and S. Hoglund. (1973). Assembly of Adenoviruses. *Journal of Virology*. 11(3): 449-459.
2. Boudin, M.-L. and P. Boulanger. (1982). Assembly of Adenovirus Penton Base and Fiber. *Virology*. 116: 589-604.
3. Rivier, J. and M. Brown. (1978). Bombesin, Bombesin Analogues, and Related Peptides: Effects on Thermoregulation. *Biochemistry*. 17(9): 1766-1771.
4. Kane, M., S. Aguay, L. Portanova, S. Ross, M. Holley, K. Kelley, and Y. Miller. (1991). Isolation of the Bombesin/Gastrin-Releasing Peptide Receptor from Human Small Cell Lung Carcinoma NCI-H345 Cells. *Journal of Biological Chemistry*. 266: 9486-9493.
5. Roux, P., P. Jeanteur, and M. Piechaczyk. (1989). A Versatile and Potentially General Approach to the Targeting of Specific Cell Types by Retroviruses: Application to the Infection of Human Cells by Means of Major Histocompatibility Complex Class I and Class II Antigens by Mouse Ecotropic Murine Leukemia Virus-Derived Viruses. *PNAS*. 86: 9079-9083.
6. Etienne-Julan, M., P. Roux, S. Carillo, P. Jeanteur, and M. Piechaczyk. (1992). The Efficiency of Cell Targeting by Recombinant Retroviruses Depends on the Nature of the Receptor and the Composition of the Artificial Cell-Virus Linker. *Journal of General Virology*. 73: 3251-3255.
7. Deshane, J., F. Loechel, R. Conry, G. Siegal, C. King, and D. Curiel. (1994). Intracellular Single-Chain Antibody Directed Against erbB2 Down-Regulates Cell Surface erbB2 and Exhibits a Selective Anti-Proliferative Effect in erbB2 Overexpressing Cancer Cell Lines. *Gene Therapy*. 1: 332-337.
8. Neda, H., C. Wu, and C. Wu. (1991). Chemical Modification of an Ecotropic Murine Leukemia Virus Results in Redirection of Its Target Cell Specificity. *Journal of Biological Chemistry*. 266(22): 14143-14146.
9. *Avidin-Biotin Technical Section,* in *Pierce Catalog and Handbook,* P.C. Company, Editor. 1994, p. T123-T125.